BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Eur. J. Biochem. 217, 469 -477 (1993)

The role of the NAD-dependent glutamate dehydrogenase in restoring growth on glucose of a *Saccharomyces cerevisiae* phosphoglucose isomerase mutant

Eckhard BOLES, Wolfgang LEHNERT and Friedrich K. ZIMMERMANN
Institut für Mikrobiologie, Technische Hochschule Darmstadt, Germany

(Received June 9, 1993) · EJB 93 0860/6

Phosphoglucose isomerase *pgi1*-deletion mutants of *Saccharomyces cerevisiae* cannot grow on glucose as the sole carbon source and are even inhibited by glucose. These growth defects could be suppressed by an over-expression on a multi-copy plasmid of the structural gene *GDH2* coding for the NAD-dependent glutamate dehydrogenase. *GDH2* codes for a protein with 1092 amino acids which is located on chromosome XII and shows high sequence similarity to the *Neurospora crassa* NAD−glutamate dehydrogenase. Suppression of the *pgi1* deletion by over-expression of *GDH2* was abolished in strains with a deletion of the glucose-6-phosphate dehydrogenase gene *ZWF1* or gene *GDH1* coding for the NADPH-dependent glutamate dehydrogenase. Moreover, this suppression required functional mitochondria. It is proposed that the growth defect of *pgi1* deletion mutants on glucose is due to a rapid depletion of NADP which is needed as a cofactor in the oxidative reactions of the pentose phosphate pathway. Over-expression of the NAD-dependent glutamate dehydrogenase leads to a very efficient conversion of glutamate with NADH generation to 2-oxoglutarate which can be converted back to glutamate by the NADPH-dependent glutamate dehydrogenase with the consumption of NADPH. Consequently, over-expression of the NAD-dependent glutamate dehydrogenase causes a substrate cycling between 2-oxoglutarate and glutamate which restores NADP from NADPH through the coupled conversion of NAD to NADH which can be oxidized in the mitochondria. Furthermore, the requirement for an increase in NADPH consumption for the suppression of the phosphoglucose isomerase defect could be met by addition of oxidizing agents which are known to reduce the level of NADPH.

Glycolysis plays a fundamental role in the degradation of fermentable carbon sources in the yeast *Saccharomyces cerevisiae* providing the cell with metabolic energy and intermediates. Phosphoglucose isomerase is the second enzyme in the glycolytic pathway, interconverting glucose 6-phosphate and fructose 6-phosphate. Maitra (1971) was the first to obtain mutants of the structural gene *PGI1* and found that they could not grow on glucose. Aguilera (1986) confirmed this for a deletion mutant with no residual activity. This was an unexpected result because the direct oxidation of glucose 6-phosphate via the pentose phosphate pathway should allow to supply yeast cells with sufficient energy and intermediate metabolites to support at least slow growth. Phosphoglucose-isomerase-lacking mutants of *Kluyveromyces lactis* (Goffrini et al., 1991) and also of *Escherichia coli* (Vinopal et al., 1975) can still grow on glucose.

On the other hand, phosphoglucose isomerase deletion mutants of *S. cerevisiae* cannot grow on a pure fructose medium because the phosphoglucose isomerase reaction is the only step catalysing the interconversion of fructose 6-phosphate to glucose 6-phosphate which is an essential metabolite (Aguilera, 1986; Boles et al., 1993). *pgi1* mutants can grow on media containing fructose and not more than 0.2% glucose, higher glucose concentrations inhibit growth (Maitra, 1971; Ciriacy and Breitenbach, 1979; Aguilera, 1986). Maitra (1971) assumed that growth inhibition results from an accumulation of toxic concentrations of glucose 6-phosphate. Ciriacy and Breitenbach (1979) suggested that ATP depletion is the cause for the glucose sensitivity.

Aguilera (1987) selected *pgi1* suppressor mutants restoring growth on synthetic media with 2% glucose as the sole carbon source. Recently, Gamo et al. (1993) isolated *pgi1* suppressor mutants insensitive to glucose inhibition by selection on media containing 2% fructose and 2% glucose. Suppression of the *pgi1* defect depended on a functional respiratory system. In both cases, the suppressor mutations were interpreted to allow *S. cerevisiae pgi1* mutant strains to enhance glucose catabolism through the pentose phosphate pathway and a complete respiratory breakdown. However, the actual functions of the suppressor genes were not identified.

Correspondence to F. K. Zimmermann, Institut für Mikrobiologie, Technische Hochschule Darmstadt, Schnittspahnstr. 10, D-64287 Darmstadt, Germany
Fax: +49 6151 164808.

Abbreviations. Glc6PDH, glucose-6-phosphate dehydrogenase; NAD-GluDH and NADPH-GluDH, NAD- and NADPH-dependent glutamate dehydrogenase; PCR, polymerase chain reaction; ORF, open reading frame.

Enzymes. Phosphoglucose isomerase (EC 5.3.1.9); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); NADPH-dependent glutamate dehydrogenase (EC 1.4.1.4); NAD-dependent glutamate dehydrogenase (EC 1.4.1.2).

Note. The nucleotide sequence of the *GDH2* gene published here has been deposited with the EMBL/GenBank sequence data bank and is available under the accession number X72015.

We prepared a yeast genomic library from a *pgi1* deletion mutant (Boles and Zimmermann, 1993a) in the multi-copy vector YEplac181 (Gietz and Sugino, 1988), transformed it back into the deletion mutant and tested the transformants for growth on a mineral salts/glucose medium. One of the glucose-positive transformants carried the structural gene *GDH2* coding for the NAD-dependent glutamate dehydrogenase. Further investigations showed that this over-expression creates a cyclic transhydrogenase system between the NADPH- and the NAD-dependent glutamate dehydrogenases, converting NADPH to NADH and replenishing the pool of NADP which is required for the oxidative reactions of the pentose phosphate pathway.

MATERIALS AND METHODS

Yeast strains and growth conditions

The isogenic wild-type strains ENY. WA-1A (*MATa, ura3-52, leu2-3,112, trp1-289, his3-delta1, MAL2-8^c, MAL3, SUC3*) and ENY. WA-1B (*MATa, ura3-52, leu2-3,112, trp1-289, HIS3, MAL2-8^c, MAL3, SUC3*) were kindly provided by K. D. Entian (University of Frankfurt). All mutants used in this work were derived from these strains unless otherwise stated. Strains EBY23 (*pgi1Δ::URA3*) and EBY8 (*fba1Δ::URA3*) were described by Boles and Zimmermann (1993a), EBY812 (*pfk1Δ::LEU2Δ, pfk2::URA3*) by Boles et al. (1993) and EBY88 (*tpi1Δ::HIS3*), EBY71 (*pgk1Δ::LEU2*), EBY66 (*gpm1Δ::LEU2*), EBY55 (*pyk1Δ::LEU2*) by Boles and Zimmermann (1993b). Strain EBY. UTL-23 (*pgi1Δ::URA3*) was derived from strain UTL-7A (*MATa, leu2-3,112, ura3-52, trp1*) (Corominas et al., 1992). Yeast cells were grown at 28°C in yeast/peptone media (1% yeast extract, 2% bactopeptone), in synthetic minimal media [0.17% Difco yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, 10 mM KH_2PO_4, pH 6.2, supplemented for auxotrophic demands] or synthetic complete media with different carbon sources (Boles and Zimmermann, 1993a, b).

Molecular biology techniques

DNA was prepared and manipulated according to the procedures described in Sambrook et al. (1989). Yeast-specific techniques were described by Guthrie and Fink (1991). pUC18, pUC19 (Yanisch-Perron et al., 1985), pBluescript II SK+ (Stratagene GmbH) and the plasmids YEplac181, YEplac112 and YEplac195 from the series of Gietz and Sugino (1988) served as vectors. *Escherichia coli* strains JM101 and SURE (Stratagene GmbH) were used for the propagation of plasmids. Plasmids were transformed into yeast according to Schiestl and Gietz (1989) and re-isolated from yeast as previously described by Boles and Zimmermann (1993a).

Construction of a genomic gene library

Yeast chromosomal DNA was prepared from strain EBY23 (*pgi1Δ*) according to Ciriacy and Williamson (1981). Chromosomal DNA (100 µg) was partially digested with 3 units of restriction enzyme *Sau*3A for 1 min at 37°C. DNA restriction fragments were electrophoresed in an agarose gel and fragments with a size between 4.5 and 10 kb were isolated from the gel. Isolated DNA fragments (9 µg) were ligated with 1 µg *Bam*HI-digested vector YEplac181 which had been dephosphorylated using calf intestinal phosphatase (Boehringer, Mannheim). The ligation reaction was transformed into *E. coli* strain SURE cells which were plated onto selective media with ampicillin, isopropyl -thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside to allow blue-white screening. There were 5000 white among a total of 11000 colonies which were washed from the plates and plasmids prepared using three Qiagen tip100 resins (Diagen GmbH).

Chromosome mapping

Gel blots with chromosome-size DNAs of *S. cerevisiae* strain FY23/6 separated in a pulsed-field gel were obtained from Boehringer (Mannheim).

DNA sequencing

Double-stranded templates were sequenced by the dideoxynucleotide chain-terminating method (Sanger et al., 1977) using the Pharmacia T7 sequencing kit. To obtain overlapping sequencing priming sites throughout the whole sequence of the insert of YEpMSP3, the transposon-based DNA sequencing strategy described by Strathmann et al. (1991) was employed using the TN1000 kit obtained from Angewandte Gentechnologie Systeme GmbH. The sequence of the complete 4.8-kb insert of YEpMSP3 was determined for both strands. The DNASIS/PROSIS program (Pharmacia) was used for DNA and protein analysis. Sequence comparisons were made by using CD-ROM and the HIBIO™ gene/protein sequence database (Hitachi).

Cloning of the *URE2* gene

The *URE2* gene was cloned by the polymerase chain reaction (PCR; Saiki et al., 1985) using a pair of primers (Roth) designed to PCR-amplify a DNA fragment enclosing part of the coding region of the *URE2* gene. One oligonucleotide (5'-AACAATAACAGCGGCCG-3') is located at position 359–375 of the published sequence (Coschigano and Magasanik, 1991) just upstream of a *Not*I restriction site. The second oligonucleotide (5'-ATGCGAATTCTATCCACGAC-ATT-3') is located at position 1155–1133. It contains a single G→C base exchange at the fifth position as compared to the published sequence to create an *Eco*RI restriction site. PCR with *Taq* polymerase (Boehringer, Mannheim) with this oligonucleotide pair as primers and chromosomal DNA of strain ENY. WA-1A as template yielded a 0.8-kb fragment containing part of the coding region of *URE2*. The fragment was then digested with *Eco*RI and *Not*I and cloned into the pBluescript II SK+ vector, resulting in plasmid pURE2.

Construction of plasmids

YEpMSP3-T: a 4.8-kb *Sst*I–*Xba*I DNA fragment of plasmid YEpMSP3 containing the complete insert was recloned into vector YEplac112.

YEpmsp3-B: a 2.9-kb *Bam*HI–*Sph*I DNA fragment containing only the first 2948 bp of the insert of plasmid YEpMSP3 was subcloned into YEplac181.

YEpmsp3-X: a 3.9-kb *Xho*I–*Sst*I fragment containing the last 3931 bp of the insert of plasmid YEpMSP3 was subcloned into YEplac181.

YEpGDH1-U: a 2.4-kb *Pst*I–*Cla*I fragment of plasmid pYC1 (Moye et al., 1985) containing the complete *GDH1* gene was subcloned into YEplac195.

pURE2del (*ure2Δ::LEU2*): the 0.3-kb *Sph*I−*Sca*I fragment of plasmid pURE2 containing part of the *URE2* coding region from +392 bp to +670 bp according to the published sequence (Coschigano and Magasanik, 1991) was replaced by a 2.5-kb *Sph*I−*Sma*I fragment with the *LEU2* gene originally cloned into pUC18.

pGDH1del (*gdh1Δ::URA3*): a 5.4-kb *Bam*HI−*Cla*I fragment of plasmid pYC1 (Moye et al., 1985) containing the *GDH1* gene was subcloned into pBluescript II SK+, resulting in pGDH1. A 1.0-kb *Hin*dIII−*Stu*I fragment of pGDH1 containing most parts of the *GDH1* coding region from +23 bp to +976 bp according to the published sequences (Moye et al., 1985; Nagasu and Hall, 1985) was replaced by a 1.1-kb *Hin*dIII−*Sma*I fragment with the *URA3* gene of plasmid YEp24 (Botstein et al., 1979).

Construction of deletion strains

The deletion mutants were constructed following the one-step gene replacement procedure of Rothstein (1983). The deletion plasmids carried different selectable markers which allowed us to construct multiple deletions in the same recipient yeast strain. pEB22 (*pgi1Δ::TRP1*) (Boles et al., 1993) was digested with *Bam*HI and *Sac*I and transformed into the haploid strain ENY. WA-1B selecting for tryptophan prototrophy on a 2% fructose and 0.1% glucose medium, resulting in strain EBY22 (*pgi1Δ::TRP1*). The deletion plasmid pM19-7 (*zwf1Δ::URA3*) of Thomas et al. (1991), which contains most parts of the open reading frame of the *ZWF1* gene replaced by the *URA3* gene, was cut by *Hin*dIII and *Bam*HI and used to transform strain EBY22 to uracil prototrophy on a 2% fructose and 0.05% glucose medium, resulting in strain EBY228 (*pgi1Δ::TRP1, zwf1Δ::URA3*). pURE2del (*ure2Δ::LEU2*) was digested with *Not*I and *Eco*RI (partially) and transformed into strain EBY22 selecting for leucine prototrophy on a 2% fructose and 0.05% glucose medium, resulting in strain EBY229 (*pgi1Δ::TRP1, ure2Δ::LEU2*). pGDH1del (*gdh1Δ::URA3*) was digested with *Pvu*II and *Cla*I and used to transform the haploid wild-type strain ENY. WA-1A to uracil prototrophy, resulting in strain EBY99 (*gdh1Δ::URA3*). Strain EBY99 was then crossed with strain EBY22 and tetrad analysis was performed, resulting in strain EBY227 (*pgi1Δ::TRP1, gdh1Δ::URA3*) which did grow on a 2% fructose and 0.05% glucose medium. Deletion mutants were confirmed by Southern blot analysis, enzyme activity assay and their growth properties.

Enzyme assays

Crude extracts were prepared using glass beads for breaking the cells as described by Ciriacy and Breitenbach (1979). Phosphoglucose isomerase activity was measured according to Maitra and Lobo (1971) and glucose-6-phosphate dehydrogenase (Glc6PDH) activity according to Kuby and Noltman (1966). NADPH-specific glutamate dehydrogenase (NADPH-GluDH) and NAD-specific glutamate dehydrogenase (NAD-GluDH) activities were assayed by using the methods of Doherty (1970) and Corman and Inamdar (1970), respectively, except that both enzyme activities were assayed in 50 mM imidazole pH 7.5, containing 10 mM $MgCl_2$, 100 mM KCl and 0.1 mM EDTA. Protein was determined as described by Zamenhof (1957) using bovine serum albumin as a standard.

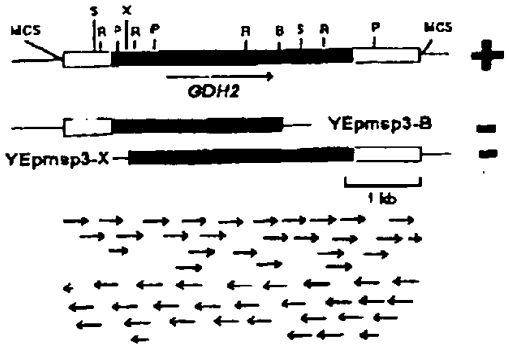

Fig. 1. Restriction map of YEpMSP3 and derivatives and the sequencing strategy. The boxes indicate yeast sequences and the lines indicate YEplac181 vector sequences. The black box indicates the open reading frame of *GDH2*. Suppression results for the deletion derivatives of YEpMSP3 are indicated at the right side: (+), suppression positive; (−) suppression negative. Little arrows mark the direction and length of the sequencing reactions. Abbreviations: B, *Bam*HI; P, *Pst*I; R, *Eco*RI; S, *Sal*I; X, *Xho*I; MCS, multiple cloning site.

Determination of metabolites

Preparation of metabolite extracts was performed essentially as described by Boles and Zimmermann (1993a) and Boles et al. (1993). Metabolite concentrations were determined according to Bergmeyer (1974).

RESULTS

Isolation of multi-copy suppressors

A genomic library in the 2μ-based multi-copy plasmid YEplac 181 (Gietz and Sugino, 1988) was prepared from a partial *Sau*3A digest of DNA of *pgi1* deletion strain EBY23 (Boles and Zimmermann, 1993a) and transformed into this strain. Transformed cells were first plated on a leucine-free medium to select for plasmid uptake and supplemented with 2% fructose and 0.1% glucose to allow all transformants to grow. More than 6000 transformant colonies grew up within four days at 28°C and were replicated onto the same basic medium supplemented with 0.1% glucose as the only carbon source. The replicas of 37 colonies had grown within two weeks. Three of these transformants lost the ability to grow on a medium with 0.1% glucose after 10−15 cell divisions on a nonselective yeast/peptone medium with 2% fructose and 0.1% glucose. This indicated that growth on glucose as the sole carbon source was due to the presence of a multi-copy plasmid-borne suppressor gene. The plasmid in the fastest growing transformant was called YEpMSP3 (for multi-copy suppressor of *pgi1* mutants to grow on glucose).

Characterization of YEpMSP3

Plasmid DNA was isolated from transformant MSP3 and characterized by restriction enzyme analysis (Fig. 1). The complete 4.8-kb insert of YEpMSP3 was recloned into vector YEplac112 (Gietz and Sugino, 1988) with a tryptophan marker to allow for selection of the plasmid in different tryptophan auxotrophic strains. The resulting plasmid YEpMSP3-T was transformed into other glycolysis mutant strains with deletions in the genes coding for pyruvate kinase

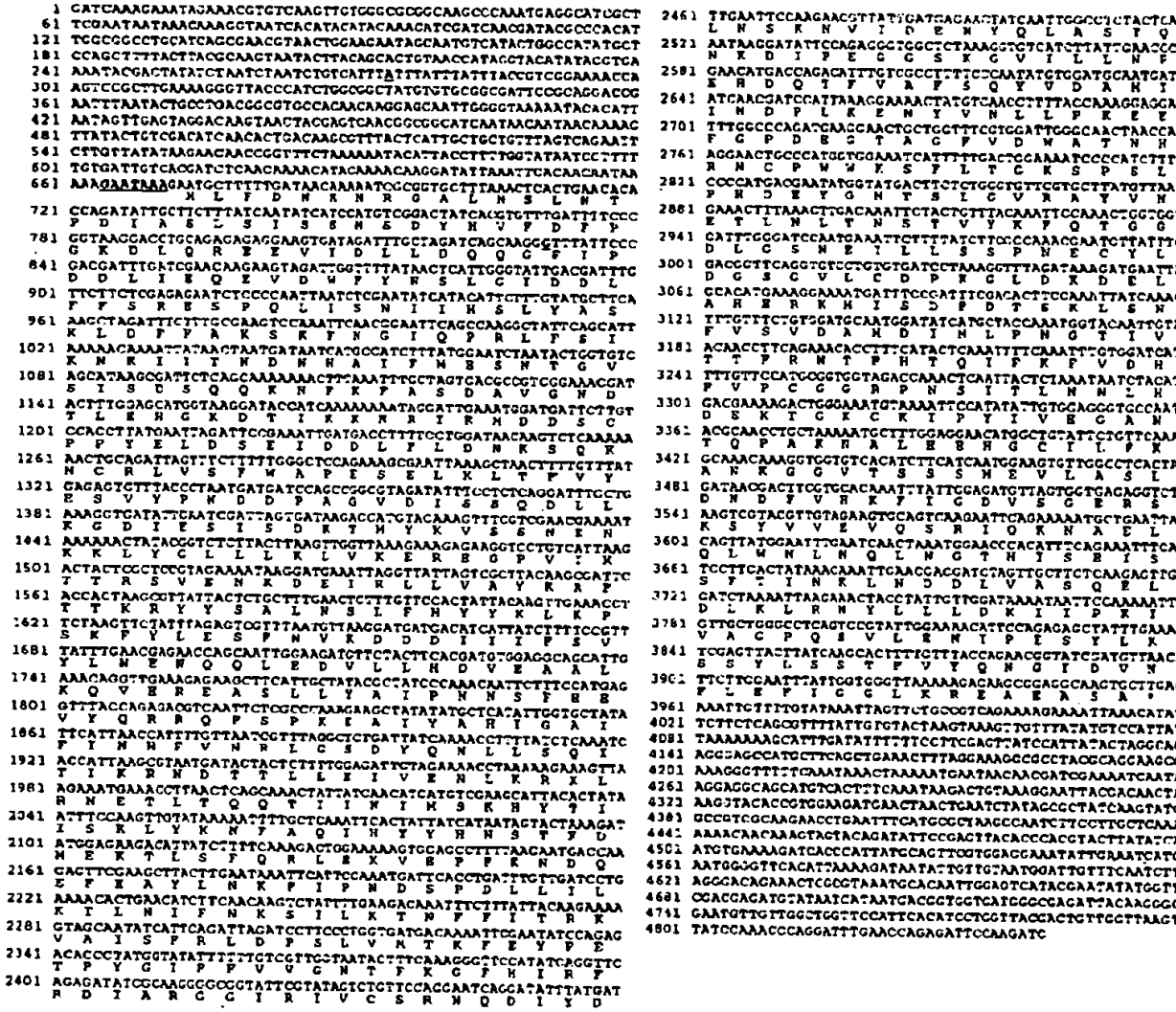

Fig. 2. Sequence of the complete insert of plasmid YEpMSP3 containing the *GDH2* gene. The predicted protein sequence produced by the *GDH2* gene, coding for the NAD-dependent glutamate dehydrogenase, is depicted below the nucleotide sequence. Numbers indicate the positions of nucleic acid residues. Differences to the sequence reported by Miller and Magasanik (1991) are printed in bold and are underlined.

(EBY55), phosphoglycerate mutase (EBY66), phosphoglycerate kinase (EBY71), triosephosphate isomerase (EBY88), fructose bisphosphate aldolase (EBY8) or phosphofructokinase (EBY812) which cannot grow on a glucose medium (Clifton et al., 1978; Ciriacy and Breitenbach, 1979; Boles and Zimmermann, 1993b). However, the ability to grow on glucose was only restored in the *pgi1* deletion strains EBY23 and EBY. UTL-23.

The nucleotide sequence of the complete 4.8-kb DNA insert was determined by sequencing of both strands (Figs 1, 2). A single complete and open reading frame from position 673 bp to 3948 bp of the insert was identified with a predicted coding capacity of 1092 amino acids of a protein of 124 kDa with a codon bias of 0.21 (Bennetzen and Hall, 1982). Suppression of the *pgi1* deletion is due to the open reading frame (ORF) and not to adjacent sequences, because deletions extending into the ORF from either direction abolished *pgi1* suppression (Fig. 1).

The nucleotide and the predicted protein sequence were compared to the databases. The DNA sequence from 2689 bp to 3607 bp still within the open reading frame was found to have about 60% similarity to a partial cDNA sequence of the *Neurospora crassa* NAD-specific glutamate dehydrogenase (GluDH) (Vierula and Kapoor, 1989). Also, at the amino acid sequence level a significant similarity was found to the 1026-amino-acid sequence of the *N. crassa* NAD-GluDH (Austen et al., 1977; Haberland and Smith, 1980) (31% identity in the amino-terminal and 54% identity in the carboxy-terminal half of the proteins). On the other hand, the *GDH2* gene of *S. cerevisiae* coding for the NAD-GluDH had been cloned and mapped by restriction enzyme analysis (Miller and Magasanik, 1990). The restriction enzyme patterns identified an

Table 1. Enzyme activities of NAD- and NADPH-dependent glutamate dehydrogenases in different strains after growth on synthetic complete media (without leucine or uracil) with ammonia as a source of nitrogen, and 2% fructose and 0.1% glucose as the carbon sources. WT = wild-type (ENY. WA-1B); *pgi1Δ* = EBY22; *pgi1Δ ure2Δ* = EBY229; YEplac181 and YEplac195 = cloning vectors; YEpMSP3 = YEplac181 with *GDH2*; YEpGDH1 = pCYG4 of Nagasu and Hall (1985) (YEp13 with *GDH1*); YEpGDH1-U = YEplac195 with *GDH1* of Moye et al. (1985).

Strain	Specific activity of	
	NAD-GluDH	NADPH-GluDH
	mU/mg protein	
WT YEplac181	9.8	236.1
pgi1Δ YEplac181	17.2	271.2
pgi1Δ YEpMSP3	4468.0	306.0
pgi1Δ YEpGDH1	19.6	3099.0
pgi1Δ ure2Δ YEplac195	340.1	158.7
pgi1Δ ure2Δ YEpGDH1-U	289.2	670.2

overlap between the *GDH2* gene and the DNA insert of YEpMSP3 which covers the complete ORF. Miller and Magasanik (1991) reported the sequence of 1300 bp of the 5′ upstream regulatory region and 239 bp of the coding region of *GDH2*. The last part of this sequence overlaps with the first 911 bp of the DNA insert of YEpMSP3 and shows 99.0% identity unequivocally identifying the suppressing gene as the *GDH2* gene of *S. cerevisiae*. Southern blot analysis confirmed that the complete insert of YEpMSP3 is an authentic fragment of yeast DNA and no rearrangements had occurred during cloning of the yeast gene or subsequent propagation of the plasmid (data not shown). We mapped the *GDH2* gene by genomic Southern blot of yeast chromosomes separated by pulse-field electrophoresis. A probe containing the complete 4.8-kb insert of YEpMSP3 cloned into the pBluescript II SK+ vector hybridized strongly only to chromosome number XII (data not shown).

Characterization of the transformants

The *pgi1* deletion strain EBY22 with plasmid YEpMSP3 (with *GDH2*) showed a 260-fold over-expression of NAD-GluDH (Table 1) as compared to strain EBY22 with plasmid YEplac181 (without the *GDH2* gene).

The *pgi1* deletion strain transformed with YEpMSP3 grew on complex yeast/peptone and the mineral salts synthetic complete and synthetic minimal media supplemented with 0.1% glucose (Fig. 3), 1% glucose, and 2% fructose in combination with 2% glucose. Doubling times in a liquid mineral salts synthetic minimal medium supplemented with 0.1% glucose were 2.5 h for wild type and 6−10 h for the transformed *pgi1* deletion strain; however, no phosphoglucose isomerase activity was detectable in crude extracts, nor did transformant strains grow on a mineral salts medium with only fructose.

The respiratory inhibitor antimycin A blocked the growth of the YEpMSP3 transformants on glucose-containing media. Therefore, suppressor activity depended on a functional respiratory system as already noted for the chromosomal suppressor mutants of Aguilera (1987) and Gamo et al. (1993).

Proposal of a model for suppression of *pgi1*

The physiological properties of the *pgi1* deletion strain with the *GDH2* over-expressed on a multi-copy vector can be explained by the following model (Fig. 4). In the first moments after addition of glucose to *pgi1* deletion cells, glucose is oxidatively degraded to ribulose 5-phosphate with the concomitant reduction of NADP to NADPH in the reactions catalyzed by glucose-6-*P* and 6-phosphogluconate dehydrogenases. This causes a rapid decrease in the level of NADP which cannot be regenerated from NADPH rapidly enough so that glucose 6-phosphate can no longer be degraded. Overexpression of the catabolic NAD-GluDH causes an elevated oxidative deamination of glutamate to 2-oxoglutarate with a simultaneous reduction of NAD to NADH. Normally, *pgi1* mutant cells exhibit very low NAD-GluDH levels in a glucose/ammonia medium (Table 1). Reductive amination of 2-oxoglutarate to glutamate by the NADPH-GluDH generates

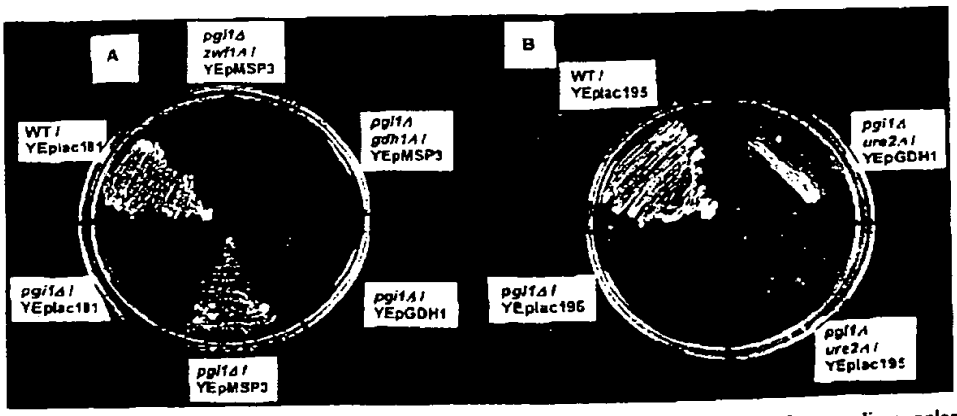

Fig. 3. Growth properties of different mutant strains. Cells were streaked out on synthetic complete media supplemented with 0.1% glucose as the carbon source and lacking leucine (A) or uracil (B). Agar plates were incubated at 28°C for 3 days (A) or 7 days (B). Strains: WT = wild-type ENY. WA-1B; *pgi1Δ* = EBY22; *pgi1Δ /gdh1Δ* = EBY227; *pgi1Δ /zwf1Δ* = EBY228; *pgi1Δ /ure2Δ* = EBY229. The strains were transformed with different plasmids: YEplac181 and YEplac195 = cloning vectors; YEpMSP3 = YEplac181 with *GDH2*; YEpGDH1 (A) = pCYG4 of Nagasu and Hall (1985) (YEp13 with *GDH1*) and YEpGDH1 (B) = YEplac195 with *GDH1* of Moye et al. (1985) (YEpGDH1-U).

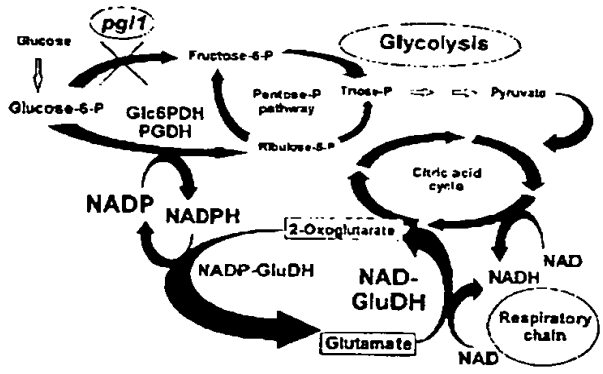

Fig. 4. Scheme of the proposed model for *pgi1* suppression by high-level expression of NAD-GluDH. See text for further information.

NADP required for the catabolism of glucose 6-phosphate to ribulose 5-phosphate. NAD can be regenerated from NADH by the respiratory system whose function is required for the suppression of the *pgi1* deletion defect. Thus, over-expression of *GDH2* creates a cyclic transhydrogenase system which oxidizes NADPH to NADP under generation of NADH.

Additional experimental support of the model

Determination of metabolites confirmed the assumptions (Table 2). After incubation with 0.1% glucose, the levels of nearly all glycolytic metabolites, 6-phosphogluconate, sedoheptulose-7-*P*, glutamate, 2-oxoglutarate and ATP were elevated in a *pgi1* mutant if transformed with YEpMSP3. However, in most cases they were still lower as in the wild-type cells. Interestingly, NADP levels were below the level of detection in the *pgi1* deletion mutant, while over-expression of *GDH2* increased the level of NADP to about half of wild-type levels. Glucose 6-phosphate concentrations were lower in the transformants but still very high as compared to the wild-type cells.

Interruption of the glutamate dehydrogenase cycle by deleting the *GDH1* gene coding for the NADPH-GluDH in a *GDH2* over-expressing *pgi1* mutant strain blocked growth on a pure glucose medium (Fig. 3) when replica-plated from permissive synthetic complete medium without leucine and with 2% fructose and 0.01% glucose. *gdh1* deletion mutant cells exhibited only slightly reduced growth rates in a wild-type background. Growth was also slightly reduced in a *pgi1* mutant background with 2% fructose plus very low amounts of glucose (data not shown) but the double deletion mutant was still more sensitive to low amounts of glucose and inhibited by glucose concentrations higher than 0.06% in combination with fructose whereas the *pgi1* single deletion mutant under the same conditions was only inhibited by glucose concentrations higher than 0.15%.

The model proposes that over-expression of *GDH2* only relieves the inhibitory effects of the glucose and does not restore a conversion of the hexose 6-phosphates by other reactions but only provides for a transhydrogenase system. The structural gene for glucose 6-phosphate dehydrogenase *ZWF1* can be deleted without any obvious metabolic defects in glucose catabolism (Nogae and Johnston, 1990; Thomas et al., 1991). A double deletion mutant *pgi1/zwf1* (strain EBY228) was inhibited on a fructose medium by glucose concentrations higher than 0.05%. Furthermore, over-expression of *GDH2* on plasmid YEpMSP3 did not restore growth on a pure glucose medium (Fig. 3).

The *URE2* gene product is a negative regulator of *GDH2* transcription (Drillien and Lacroute, 1972; Drillien et al.,

Table 2. Concentrations of metabolites. Cells of strains ENY. WA-1B (wild-type) and EBY22 (*pgi1Δ*) were grown overnight in a yeast/peptone medium with 2% fructose and 0.1% glucose, washed once with water and shifted into yeast/peptone medium supplemented with 0.1% glucose 2 h before preparation of extracts. Cells of strain EBY22 carrying the multi-copy plasmid YEpMSP3 with the *GDH2* gene (*pgi1Δ*/YEpMSP3) were first grown overnight in yeast/peptone medium with 0.1% glucose, washed once with water and then shifted into a yeast/peptone/0.1% glucose medium 2 h before preparation of the extract.

Metabolite	Concentration in		
	WT (ENY. WA-1B) 0.1% glucose	*pgi1Δ*/YEpMSP3 0.1% glucose	*pgi1Δ* (EBY22) 0.1% glucose
	nmol/mg dry mass		
Glucose-6-*P*	2.07	58.60	76.20
Gluconate-6-*P*	0.16	1.09	0.27
Sedoheptulose-7-*P*	0.52	0.39	< 0.10
Fructose-6-*P*	0.43	< 0.10	< 0.10
Fructose-1,6-P_2	4.99	0.81	0.25
Triose-*P*	0.73	0.50	0.13
Glycerate-3-*P*	1.34	1.01	0.38
Glycerate-2-*P*	0.18	0.33	0.21
Phospho*enol*pyruvate	0.29	0.66	0.26
Pyruvate	0.74	0.69	0.36
2-Oxoglutarate	1.43	1.28	0.83
Glutamate	74.27	31.16	21.52
ATP	5.30	3.56	0.87
NAD	3.69	4.96	3.91
NADH	3.18	4.09	3.38
NADP	0.25	0.14	< 0.10
NADPH	0.70	0.98	0.77

1973; Coschigano and Magasanik, 1991). In *pgi1/ure2* double deletion mutants NAD-GluDH activity was strongly elevated but, at the same time, NADPH-GluDH activity was reduced to about one half of the value of the *pgi1* single deletion mutant (Table 1). *pgi1/ure2* double deletion mutants could not grow on a pure glucose medium (Fig. 3) but increasing the level of the NADPH-dependent GluDH by transformation with plasmid YEpGDH1-U (Table 1) enabled the double mutant cells to grow very slowly on 0.1% glucose (Fig. 3). On the other hand, a more than tenfold elevated NADPH-GluDH level in the *pgi1* single deletion mutant EBY22 by transformation with plasmid pCYG4 carrying *GDH1* (Nagasu and Hall, 1985) did not at all restore growth on pure glucose medium (Table 1, Fig. 3).

The model suggested that conditions causing an increased oxidation of NADPH will also suppress the phosphoglucose isomerase defect. Oxidizing agents are known to reduce the level of NADPH, presumably via the reaction of glutathione reductase (Kosower and Kosower, 1969; see Nogae and Johnston, 1990). For the experimental generation of oxidative stress, H_2O_2 or electron-transferring chemicals, such as menadione, which transfer electrons from a cellular donor to molecular oxygen (Hassan and Fridovich, 1979) can be used (Schnell et al., 1992). Actually, addition of H_2O_2 (not shown) or menadione promoted growth of *pgi1* mutant cells on a pure glucose medium (Fig. 5). Furthermore, menadione could not promote growth on glucose of a *zwf1/pgi1* double deletion strain (Fig. 5).

Fig. 5. Restoration of growth of *pgi1* mutant cells by menadione. Growth on yeast/peptone supplemented with 0.2% glucose of (A) wild-type (WT) strain (ENY. WA-1B) with 5 µl 30% menadione (M) as a control; (B) the *pgi1* mutant (strain EBY22) with 5 µl 30% menadione; (C) the *pgi1* mutant (strain EBY22) with 5 µl H_2O; (D) the *pgi1/zwf1* double deletion mutant (strain EBY228) with 5 µl 30% menadione. Cells were grown first in yeast/peptone/2% fructose + 0.01% glucose liquid media and plated on to the agar plates with identical cell densities. The agar plates were incubated for 4 days at 28°C.

DISCUSSION

Efficient recycling of co-substrates is important for metabolic fluxes. In glycolysis, NAD is the co-substrate in the glyceraldehyde-3-phosphate dehydrogenase reaction and the generated NADH has to be re-oxidized in the alcohol dehydrogenase reaction. A total lack of alcohol dehydrogenase (Ciriacy, 1975) and pyruvate decarboxylase (Schmitt and Zimmermann, 1982; Hohmann and Cederberg, 1990) in *S. cerevisiae* blocks glycolysis. An unexpected result was that glycolysis in *S. cerevisiae* is also blocked in mutants lacking trehalose synthase. This was explained by Hohmann et al. (1993) who suggested that trehalose synthesis functions as a 'metabolic buffer system' to recycle the phosphate residues rapidly bound in glucose 6-phosphate after addition of glucose back to inorganic phosphate which is needed for the glyceraldehyde-3-phosphate dehydrogenase reaction.

Mutants with reduced phosphoglucose isomerase activities accumulate high concentrations of glucose 6-phosphate when supplied with glucose as already noted by Maitra (1971) who interpreted the inhibitory effect of glucose as a toxicity of glucose 6-phosphate because a limited amount of growth should be possible by the 'direct oxidation' of glucose 6-phosphate via the pentose phosphate pathway. Our data suggest that it is the rapid consumption of NADP and an insufficient rate of regeneration of NADP from NADPH which prevents the 'direct oxidation' from operating. However, establishing a transhydrogenase system by a massive over-expression of the NAD-dependent glutamate dehydrogenase or oxidative stress induced by H_2O_2 or menadione which causes a strong oxidation of NADPH suppress the inability of phosphoglucose isomerase mutant to grow on glucose. Both conditions establish a new example for a metabolic regeneration system.

NAD-GluDH serves a catabolic function catalyzing the NAD-dependent oxidative deamination of glutamate to 2-oxoglutarate and ammonium ion (reviewed by Magasanik, 1992). The gene *GDH2* coding for NAD-GluDH was cloned previously and partially sequenced by Miller and Magasanik (1990, 1991). Expression of *GDH2* is subjected to nitrogen and carbon regulation (Courchesne and Magasanik, 1988; Coschigano and Magasanik, 1991; Minchart and Magasanik, 1991; Coschigano et al., 1991). NADPH-GluDH is the key enzyme of *S. cerevisiae* in the anabolic conversion of ammonia into an organic form by a reductive amination of 2-oxoglutarate to glutamate using NADPH as the cofactor (reviewed by Cooper, 1982). The gene *GDH1* coding for NADPH-GluDH had been cloned and sequenced by Moye et al. (1985) and Nagasu and Hall (1985). NADPH-GluDH levels are high and NAD-GluDH levels are low when ammonium ions are supplied as nitrogen source.

The low wild-type level of NAD-GluDH activity was also found in a *pgi1* deletion mutant incubated in a fructose/glucose/ammonia medium (Table 1). It could be increased either by transformation into the cells of a multi-copy plasmid carrying the *GDH2* gene or by deletion of the *URE2* gene which codes for a negative regulator of *GDH2* expression (Coschigano and Magasanik, 1991). The nearly 300-fold elevated level of NAD-GluDH in the transformants is probably due to a deregulation of the gene if present on a

multi-copy plasmid and an elevated copy number of the plasmid due to selective pressure for very high NAD-GluDH activities. In both cases, growth could only be restored in the presence of sufficient NADPH-GluDH activity. The alternative pathway for the net biosynthesis of glutamate from 2-oxoglutarate by glutamine synthetase and glutamate synthase cannot substitute NADPH-GluDH for providing NADP because the glutamate synthase of *S. cerevisiae* uses NADH as its cofactor (Roon et al., 1974). These facts clearly demonstrate the necessity of a substrate cycling between the two glutamate dehydrogenases. NADPH-GluDH is primary responsible for the replenishment of the NADP pool. NAD-GluDH serves only an indirect function of further metabolizing the glutamate which has been generated by NADPH-GluDH. On the other hand, increasing the level of NADPH-GluDH in a *pgi1* single deletion mutant did not at all restore growth on glucose (Fig. 3). This can be explained by two considerations. First, a high level of NADPH-GluDH removes 2-oxoglutarate which is needed as a substrate for oxidative respiration from the citric acid cycle. Growth on pure glucose medium of *pgi1* transformants over-expressing NAD-GluDH was inhibited by antimycin A indicating that respiration is necessary for growth. Second, glutamate accumulates because it cannot be metabolized fast enough by other reactions and the reversible NADPH-GluDH reaction will reach a steady-state equilibrium.

Furthermore, NADPH-GluDH and NAD-GluDH are both cytosolic enzymes (Hollenberg et al., 1970; Perlman and Mahler, 1970). For this reason no NADP shuttle is needed for the metabolic interplay between the pentose phosphate pathway and the glutamate dehydrogenase cycle.

A *pgi1* deletion mutant is inhibited by glucose concentrations higher than 0.15% on a 2% fructose medium. This type of glucose sensitivity could also be overcome by *pgi1* transformants over-expressing *GDH2*. On the other hand, *pgi1/zwf1* and to a lesser extent *pgi1/gdh1* double deletion mutants were even more sensitive to trace amounts of glucose added together with the fructose than *pgi1* single mutants. Therefore, NADP depletion cannot be the true reason for glucose sensitivity. Our data cannot unequivocally decide whether accumulation of toxic concentrations of glucose 6-phosphate (Maitra, 1971) or depletion of ATP (Ciriacy and Breitenbach, 1979) is the reason for glucose sensitivity but it is interesting to note that *pgi1* mutants over-expressing *GDH2* could grow on glucose with doubling times only about three times higher than wild-type cells although glucose 6-phosphate was present at very high levels (Table 2). Also, the strong accumulation of hexose phosphates generated in the glycolytic and the pentose phosphate pathways and the depletion of ATP and not a depletion of NADP seem to be the reason for the growth deficiencies on glucose of glycolysis mutants which are blocked in the reactions of phosphofructokinase, fructose bisphosphate aldolase or triosephosphate isomerase because in those mutants growth could not be restored by high-level expression of *GDH2*.

Phosphoglucose-isomerase-negative mutants of *E. coli* (Fraenkel and Levisohn, 1967) and of *K. lactis* (Goffrini et al., 1991) are able to grow on glucose medium, although at a reduced rate. Apparently, they utilize glucose primarily by the pentose phosphate pathway. Csonka and Fraenkel (1977) observed that *E. coli* mutants lacking both phosphoglucose isomerase and the membrane ATPase could no longer grow on glucose. They suggested that in a phosphoglucose isomerase mutant the energy-linked transhydrogenase might be used in NADPH oxidation. Lagunas and Gancedo (1973) supposed that a significant transhydrogenation between pyridine nucleotides is not likely to occur in *S. cerevisiae* growing on glucose. This fact could explain the different properties of phosphoglucose isomerase mutants of different species.

We thank H. Zalkin and B. D. Hall for kind provision of the *GDH1* genes, Y. Surdin-Kerjan for provision of the *zwf1* deletion plasmid and K.-D. Entian for provision of the yeast strains ENY. WA-1A and ENY. WA-1B. The work in our laboratory was partly supported by the *Deutsche Forschungsgemeinschaft*.

REFERENCES

Aguilera, A. (1986) Deletion of the phosphoglucose isomerase structural gene makes growth and sporulation glucose dependent in *Saccharomyces cerevisiae*, *Mol. Gen. Genet. 204*, 310–316.

Aguilera, A. (1987) Mutations suppressing the effect of a deletion of the phosphoglucose isomerase gene *PGI1* in *Saccharomyces cerevisiae*, *Curr. Genet. 11*, 429–434.

Austen, B. M., Haberland, M. E., Nyc, J. F. & Smith, E. L. (1977) Nicotinamide adenine dinucleotide-specific glutamate dehydrogenase of *Neurospora*, *J. Biol. Chem. 252*, 8142–8149.

Bennetzen, J. L. & Hall, B. D. (1982) Codon selection in yeast, *J. Biol. Chem.257*, 3026–3031.

Bergmeyer, H. U. (1974) Methoden der enzymatischen Analyse, Verlag Chemie, Weinheim.

Boles, E., Heinisch, J. & Zimmermann, F. K. (1993) Different signals control the activation of glycolysis in the yeast *Saccharomyces cerevisiae*, *Yeast 9*, 761–770.

Boles, E. & Zimmermann, F. K. (1993a) *Saccharomyces cerevisiae* phosphoglucose isomerase and fructose bisphosphate aldolase can be replaced functionally by the corresponding enzymes of *Escherichia coli* and *Drosophila melanogaster*, *Curr. Genet. 23*, 187–191.

Boles, E. & Zimmermann, F. K. (1993b) Induction of pyruvate decarboxylase in glycolysis mutants of *Saccharomyces cerevisiae* correlates with the concentrations of three-carbon glycolytic metabolites, *Arch. Microbiol.*, in the press.

Botstein, D., Falco, S. C., Dtewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K. & Davis, R. W. (1979) Sterile host yeast (SIIY): a eukaryotic system of biological containment for recombinant DNA experiments, *Gene 8*, 17–24.

Ciriacy, M. (1975) Genetics of alcohol dehydrogenase in *Saccharomyces cerevisiae*. I. Isolation and genetic analysis of *adh* mutants, *Mutat. Res. 29*, 315–326.

Ciriacy, M. & Breitenbach, I. (1979) Physiological effects of seven different blocks in glycolysis in *Saccharomyces cerevisiae*, *J. Bacteriol. 139*, 152–160.

Ciriacy, M. & Williamson, V. M. (1981) Analysis of mutations affecting Ty-mediated gene expression in *Saccharomyces cerevisiae*, *Mol. Gen. Genet. 182*, 159–163.

Clifton, D., Weinstock, S. B. & Fraenkel, D. G. (1978) Glycolysis mutants in *Saccharomyces cerevisiae*, *Genetics 88*, 1–11.

Cooper, T. G. (1982) Nitrogen metabolism in *Saccharomyces cerevisiae*, in *The molecular biology of the yeast Saccharomyces. Metabolism and gene expression* (Strathern, J. N., Jones, E. W. & Broach, J. R., eds) vol. 2, pp. 39–99, Cold Spring Harbor Laboratory Press, Plainsville NY.

Corman, L. & Inamdar, A. (1970) L-*Glutamate dehydrogenase (dogfish liver and chicken liver)*, *Methods Enzymol. 17*, 844–850.

Cornminas, J., Clotet, J., Fernández-Banares, I., Boles, E., Zimmermann, F. K., Guinovart J. J. & Arino, J. (1992) Glycogen metabolism in a *Saccharomyces cerevisiae* phosphoglucose isomerase (*pgi1*) disruption mutant, *FEBS Lett. 310*, 182–186.

Coschigano, P. W. & Magasanik, B. (1991) The *URE2* gene product of *Saccharomyces cerevisiae* plays an important role in the cellular response to the nitrogen source and has homology to glutathione *S*-transferases, *Mol. Cell. Biol. 11*, 822–832.

Coschigano, P. W., Miller, S. M. & Magasanik, B. (1991) Physiological and genetic analysis of the carbon regulation of the NAD-

dependent glutamate dehydrogenase of *Saccharomyces cerevisiae*, *Mol. Cell. Biol. 11*, 4455–4465.
Courchesne, W. E. & Magasanik, B. (1988) Regulation of nitrogen assimilation in *Saccharomyces cerevisiae*: roles of the *URE2* and *GLN3* genes, *J. Bacteriol. 170*, 708–713.
Csonka, L. N. & Fraenkel, D. G. (1977) Pathways of NADPH formation in *Escherichia coli*, *J. Biol. Chem. 252*, 3382–3391.
Doherty, D. (1970) L-Glutamate dehydrogenase (yeast), *Methods Enzymol. 17*, 850–856.
Drillien, R., Aigle, M. & Lacroute, F. (1973) Yeast mutants pleiotropically impaired in the regulation of two glutamate dehydrogenases, *Biochem. Biophys. Res. Commun. 53*, 367–372.
Drillien, R. & Lacroute, F. (1972) Ureidosuccinic acid uptake in yeast and some aspects of its regulation, *J. Bacteriol. 109*, 203–208.
Fraenkel, D. G. & Levisohn, S. R. (1967) Glucose and gluconate metabolism in an *Escherichia coli* mutant lacking phosphoglucose isomerase, *J. Bacteriol. 93*, 1571–1578.
Gamo, F. J., Portillo, F. & Gancedo, C. (1993) Characterization of mutations that overcome the toxic effect of glucose on phosphoglucose isomerase less strains of *Saccharomyces cerevisiae*, *FEMS Microbiol. Lett. 106*, 233–238.
Gietz, R. D. & Sugino, A. (1988) New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites, *Gene 74*, 527–534.
Goffrini, P., Wésolowski-Louvel, M. & Ferrero, I. (1991) A phosphoglucose isomerase gene is involved in the rag phenotype of the yeast *Kluyveromyces lactis*, *Mol. Gen. Genet. 228*, 401–409.
Guthrie, C. & Fink, G. R. (ed.) (1991) Guide to yeast genetics and molecular biology, *Methods Enzymol. 194*, Academic Press, San Diego.
Haberland, M. E. & Smith, E. L. (1980) Nicotinamide adenine dinucleotide-specific glutamate dehydrogenase of *Neurospora crassa*, *J. Biol. Chem. 255*, 7984–7992.
Hassan, H. M. & Fridovich, I. (1979) Paraquat and *Escherichia coli*, *J. Biol. Chem. 254*, 10846–10852.
Hohmann, S. & Cederberg, H. (1990) Autoregulation may control the expression of yeast pyruvate decarboxylase structural genes *PDC1* and *PDC5*, *Eur. J. Biochem. 188*, 615–621.
Hohmann, S., Neves, M. J., de Koning, W., Alijo, R., Ramos, J. & Thevelein, J. M. (1993) The growth and signalling defects of the *ggs1* (*fdp1/byp1*) deletion mutant on glucose are suppressed by a deletion of the gene encoding hexokinase PII, *Curr. Genet. 23*, 281–289.
Hollenberg, C. P., Riks, W. F. & Borst, P. (1970) The glutamate dehydrogenases of yeast: extra-mitochondrial enzymes, *Biochim. Biophys. Acta 201*, 13–19.
Kosower, E. M. & Kosower, N. S. (1969) Lest I forget thee, glutathione, *Nature 224*, 117–120.
Kuby, S. A. & Noltman, E. A. (1966) Glucose 6-phosphate dehydrogenase (crystalline) from brewers' yeast, *Methods Enzymol. 9*, 116–125.
Lagunas, R. & Gancedo, J. M. (1973) Reduced pyridine-nucleotides balance in glucose-growing *Saccharomyces cerevisiae*, *Eur. J. Biochem. 37*, 90–94.
Magasanik, B. (1992) Regulation of nitrogen utilization, in *The molecular and cellular biology of the yeast Saccharomyces. Gene expression* (Jones, E. W., Pringle, J. R. & Broach, J. R., eds) vol. 2, pp. 283–317, Cold Spring Harbor Laboratory Press, Plainsville NY.
Maitra, P. K. (1971) Glucose and fructose metabolism in a phosphogluconeisomeraseless mutant of *Saccharomyces cerevisiae*, *J. Bacteriol. 107*, 759–769.
Maitra, P. K. & Lobo, Z. (1971) A kinetic study of glycolytic enzyme synthesis in yeast, *J. Biol. Chem. 246*, 475–488.
Miller, S. M. & Magasanik, B. (1990) Role of NAD-linked glutamate dehydrogenase in nitrogen metabolism in *Saccharomyces cerevisiae*, *J. Bacteriol. 172*, 4927–4935.
Miller, S. M. & Magasanik, B. (1991) Role of the complex upstream region of the *GDH2* gene in nitrogen regulation of the NAD-linked glutamate dehydrogenase in *Saccharomyces cerevisiae*, *Mol. Cell. Biol. 11*, 6229–6247.
Minehart, P. L. & Magasanik, B. (1991) Sequence and expression of *GLN3*, a positive nitrogen regulatory gene of *Saccharomyces cerevisiae* encoding a protein with a putative zinc finger DNA-binding domain, *Mol. Cell. Biol. 11*, 6216–6228.
Moye, W. S., Amuro, N., Mohana Rao, J. K. & Zalkin, H. (1985) Nucleotide sequence of yeast *GDH1* encoding nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase, *J. Biol. Chem. 260*, 8502–8508.
Nagasu, T. & Hall, B. D. (1985) Nucleotide sequence of the *GDH* gene coding for the NADP-specific glutamate dehydrogenase of *Saccharomyces cerevisiae*, *Gene 37*, 247–253.
Nogae, I. & Johnston, M. (1990) Isolation and characterization of the *ZWF1* gene of *Saccharomyces cerevisiae*, encoding glucose 6-phosphate dehydrogenase, *Gene 96*, 161–169.
Perlman, P. S. & Mahler, H. R. (1970) Intracellular localization of enzymes in yeast, *Arch. Biochem. Biophys. 136*, 245–250.
Roon, R. J., Even, H. L. & Larimore, F. (1974) Glutamate synthase: properties of the reduced nicotinamide adenine dinucleotide-dependent enzyme from *Saccharomyces cerevisiae*, *J. Bacteriol. 118*, 89–95.
Rothstein, R. J. (1983) One step gene disruption in yeast, *Methods Enzymol. 101*, 202–211.
Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. & Arnheim, N. (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia, *Science 230*, 1350–1354.
Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular cloning: a laboratory manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Plainsville NY.
Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA-sequencing with chain-terminating inhibitors, *Proc. Natl Acad. Sci. USA 74*, 5463–5467.
Schiestl, R. H. & Gietz, R. D. (1989) High efficiency transformation of intact yeast cells by single stranded nucleic acids as carrier, *Curr. Genet. 16*, 339–346.
Schmitt, H. D. & Zimmermann, F. K. (1982) Genetic analysis of the pyruvate decarboxylase reaction in yeast glycolysis, *J. Bacteriol. 151*, 1146–1152.
Schnell, N., Krems, B. & Entian, K. D. (1992) The *PAR1* (*YAP1/SNQ3*) gene of *Saccharomyces cerevisiae*, a c-*jun* homologue, is involved in oxygen metabolism, *Curr. Genet. 21*, 269–273.
Strathmann, M., Hamilton, B. A., Mayeda, C. A., Simon, M. I., Meyerowitz, E. M. & Palazzolo, M. J. (1991) Transposon-facilitated DNA sequencing, *Proc. Natl Acad. Sci. USA 88*, 1247–1250.
Thomas, D., Cherest, H. & Surdin-Kerjan, Y. (1991) Identification of the structural gene for glucose 6-phosphate dehydrogenase in yeast. Inactivation leads to a nutritional requirement for organic sulfur, *EMBO J. 10*, 547–553.
Vierula, P. J. & Kapoor, M. (1989) NAD-specific glutamate dehydrogenase of *Neurospora crassa*: cDNA cloning and gene expression during derepression, *J. Biol. Chem. 264*, 1108–1114.
Vinopal, R. T., Hillman, J. D., Schulman, H., Reznikoff, W. S. & Fraenkel, D. G. (1975) New phosphoglucose isomerase mutants of *Escherichia coli*, *J. Bacteriol. 122*, 1172–1174.
Yanisch-Perron, C., Vieira, J. & Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors, *Gene 33*, 103–119.
Zamenhoff, S. (1957) Preparation and assay of deoxyribonucleic acids from animal tissue, *Methods Enzymol. 3*, 696–704.